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Fatty Acid Production from Amino Acids and α-Keto Acids by *Brevibacterium linens* BL2†

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Low concentrations of branched-chain fatty acids, such as isobutyric and isovaleric acids, develop during the ripening of hard cheeses and contribute to the beneficial flavor profile. Catabolism of amino acids, such as branched-chain amino acids, by bacteria via aminotransferase reactions and α-keto acids is one mechanism to generate these flavorful compounds; however, metabolism of α-keto acids to flavor-associated compounds is controversial. The objective of this study was to determine the ability of *Brevibacterium linens* BL2 to produce fatty acids from amino acids and α-keto acids and determine the occurrence of the likely genes in the draft genome sequence. BL2 catabolized amino acids to fatty acids only under carbohydrate starvation conditions. The primary fatty acid end products from leucine were isovaleric acid, acetic acid, and propionic acid. In contrast, logarithmic-phase cells of BL2 produced fatty acids from α-keto acids only. BL2 also converted α-keto acids to branched-chain fatty acids after carbohydrate starvation was achieved. At least 100 genes are potentially involved in five different metabolic pathways. The genome of *B. linens* ATCC 9174 contained these genes for production and degradation of fatty acids. These data indicate that brevibacteria have the ability to produce fatty acids from amino and α-keto acids and that carbon metabolism is important in regulating this event.

Positive cheese flavor is a result of the balance between different flavor compounds that are produced during ripening. Two important classes of compounds that contribute to positive cheese flavor are volatile sulfur compounds (VSCs) and fatty acids (FAs) (7, 21). The exact concentrations of VSCs and FAs that are optimal for the best cheese flavor are variable (22), and the processing events that control flavor compound production are not well understood, despite extensive biochemical details that are well known. Catabolism of sulfur-containing amino acids by brevibacteria is characterized in limited detail, but brevibacteria produce high quantities of VSCs from methionine (3, 5) via a single catalytic step using methionine-γ-lyase, which catalyzes the conversion of methionine to methanethiol by demethiolation (4, 21).

Addition of *Brevibacterium linens* to low-fat cheddar cheese significantly enhanced the consumer flavor acceptance, largely due to sulfur metabolism (20, 21). *B. linens* accelerates the ripening process in cheddar cheese via its proteolytic capabilities, VSC production, and other unidentified pathways, including FA production (20). Production of flavor-enhancing compounds, especially branched-chain FAs (BCFAs), by lactococci and brevibacteria is poorly defined. Catabolism of branched-chain amino acids (BCAAs) to produce FAs in lactococci delineated a portion of the mechanism involved in production of these compounds that is modulated by *ilvE* (8), but the regulation of production is not fully understood. Aminotransferases (ATases) have been reported to initiate catabolism of aromatic, branched-chain, and sulfur amino acids to VSCs and FAs in lactococci (24).

Previous studies with lactic acid bacteria and brevibacteria found amino acids are readily converted to FAs (9, 13–15). *B. linens* produces acetic acid from glycine, alanine, and leucine; isovaleric acid from leucine; and caproic acid from cystine, alanine, and serine (13, 14). Interestingly, BCFAs do not occur in milk, yet BCFAs are found in fermented dairy products, highlighting the holes in the understanding of the metabolic mechanisms for FA production in these organisms, but it is certain that microbial catabolism is important in the production of FAs in dairy products (9, 12, 15).

Lactococci and lactobacilli produce FAs from amino and α-keto acid substrates (8, 9). This step is initiated by ATases that have broad substrate specificities in lactic acid bacteria (8, 9, 23, 24). Ganesan and Weimer (8) found lactococci produced BCFAs from BCAAs even if the branched-chain ATase was deleted (Δ*ilvE*) with amino or α-keto acids added as the substrate. The proposed metabolic pathways demonstrate individual pathways (9) for each amino acid substrate for BCFA production. While such a study is not available for *B. linens*, after examination of the draft genome sequence for *B. linens* ATCC 9174 (http://www.jgi.doe.gov/JGI_microbial/html/index.html) it is reasonable to suspect that this organism also has the ability to produce BCFAs and FAs. Examination of the draft sequence of the genome found 18 ATases, including two homologues of *ilvE* (EC 2.6.1.42). It also contains genes that encode α-keto acid dehydrogenases, phosphotransacylases, and acyl kinases. However, the conditions for the transcription of these enzymes are unknown.

FA production via amino acid catabolism may be beneficial to brevibacteria, as the associated pathways generate ATP via substrate-level phosphorylation and regenerate protons that

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maintain cellular oxidation-reduction potential (11). *B. linens* also possesses enzymes required for catabolism of FAs via β-oxidation and the utilization of the generated acyl-CoAs via the tricarboxylic acid cycle. Hence, the catabolism of BCAAs may be beneficial for *B. linens* to generate multiple ATPs and biosynthetic intermediates like α-keto acids via subsequent pathways. Specific details beyond the initial ATase reaction in the production of BCFAs remain to be established in brevibacteria.

Based on these studies, we hypothesized that brevibacteria generate BCFAs from amino acids and α-keto acids. To test this hypothesis, a buffer assay system was used with individual substrates and their mixtures. This study found that *B. linens* BL2 produced BCFAs from amino acids and α-keto acids at a pH of 5.2, in 4% NaCl only after carbohydrate starvation was achieved. Subsequently, the FA pool declined, suggesting that the organism metabolized the FAs via β-oxidation as an energy source.

MATERIALS AND METHODS

Strains and media. Stock cultures of *B. linens* BL2 were prepared by growing cultures twice in 100 ml of tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) at 25°C with shaking at 225 rpm for 24 to 48 h. The cultures were frozen at −70°C in TSB containing 30% glycerol. Prior to each experiment a working culture was grown from a frozen stock culture by subculturing it twice in TSB at 25°C for 48 h with shaking at 225 rpm in 100 ml of broth in a 250-ml flask. The cells from this culture were used to inoculate specific media for the test conditions after harvest from 10 ml by centrifugation (3,500 × *g* for 15 min at 4°C) and two washing steps with 10 ml of sterile 0.05 M potassium phosphate buffer (pH 7.2). These cells were resuspended in the appropriate medium or assay buffer containing the substrates described below.

FA production assays. Growth under glucose-containing conditions was done using TSB incubated at 25°C for 48 h with shaking at 225 rpm in 100 ml of medium in a 250-ml flask. Logarithmic-phase cells were assayed for FA production in assay buffer, and FAs were extracted from the spent buffer similar to the method for carbohydrate-starved cells described below, after filter sterilization with 0.2-μm-pore-size syringe filters (Nalge Company, Rochester, N.Y.) followed by gas chromatography (GC) analysis.

The cells exposed under carbohydrate-depleted conditions were grown in a chemically defined medium (CDM) (10), except the culture was incubated at 25°C with shaking at 225 rpm and sampled (10-ml aliquots) at 24-h intervals for up to 7 days. At each time point, cells from 10 ml of CDM were harvested by centrifugation (3,500 × *g* for 15 min at 4°C), washed with 10 ml of sterile 0.05 M potassium phosphate buffer (pH 7.2), and resuspended in assay buffer containing the various substrates.

Carbohydrate-starved cells from incubation in CDM were harvested, washed, and resuspended in 5 ml of 0.15 M 2-(*N*-morpholino)ethanesulfonic acid sodium salt buffer (pH 5.2) containing 4% NaCl, to a final A_{600} of 0.2. The assay buffer containing the substrate and cells was incubated at 25°C for 3 h without shaking, as previously described (13, 15, 16). Subsequently, the entire assay mixture was filter sterilized with 0.2-μm-pore-size syringe filters (Nalge Company), and the FAs were extracted for GC analysis. Additionally, the suspended cells were incubated in sterile buffer (negative control), individual amino acids, individual α-keto acids, and mixtures of the amino acids or α-keto acids. The BCAAs used were leucine and valine. The α-keto acids used were α-keto-isocaproate (KIC), α-keto-glutarate (KGL), α-keto-isovalerate (KIV), and α-keto–β-methylvalerate (KMV), individually, with pyruvate, and as a mixture. All substrates were added to a total final concentration of 1 mM substrate in the resuspended cell incubation buffer. Amino acids, α-keto acids, FA standards, and buffer salts were purchased from Sigma-Aldrich (St. Louis, Mo.). The control samples did not contain any FAs in freshly prepared solutions (9).

Studies using nuclear magnetic resonance (NMR) to confirm FA production also used CDM for carbohydrate starvation followed by incubation in the assay buffer as described above. The NMR assay mixture consisted of 20 mM L-leucine-2-^{13}C (Isotec Inc., Miamisburg, Ohio), 20 mg of washed BL2 cells grown in CDM/ml, and 30 mM sodium phosphate buffer (pH 7.0). This assay mixture was incubated at 25°C with shaking at 225 rpm and sampled at 24-h intervals for up to 7 days. Samples were centrifuged (3,500 × *g* for 15 min at 4°C), and the supernatant was frozen at −20°C until NMR analysis was done. A cell suspension with an A_{650} of 1.0 contained 0.365 mg of cells/ml of culture (6), which was used to calculate the weight of the cells.

The frozen samples were thawed and, to 0.45 ml of sample, 50 μl of deuterated water (Isotec Inc.) was added and the mixture was placed in a 5-mm NMR tube. An insert tube containing chloroform was placed inside the NMR tube to provide a standard upon which to calibrate all chemical shifts (17).

GC. FAs were extracted from the assay buffer that was filter sterilized as described previously (9). The extracted FAs were analyzed by the method described above, except 1 μl was injected by hand during GC analysis. FAs were identified and quantified by the internal standard method as described elsewhere (2, 9). Results were obtained from two replications of the assays and are expressed as the concentration (in millilolar) of FAs.

NMR spectroscopy. NMR spectroscopy was done at the Department of Chemistry and Biochemistry, Utah State University, Logan. All ^{13}C-NMR spectra were obtained with a Bruker model ARX-400 NMR spectrometer operating at a carbon NMR frequency of 100.6 MHz with a 5-mm multinuclear probe at a temperature of 27°C, as recommended by the Bruker pulse program zgdc, with the following parameters: ^{13}C spectral window, 225 ppm; 90° pulse width, 8 ms; relaxation delay, 1 s; scans per spectrum, 3,200; total acquisition time, 2 h. Deuterated water was used to obtain the signal lock. In a 7-day sample, a 30,000-scan spectrum with a 20-h total acquisition time was done to identify unknown peaks. All NMR spectra were referenced to an internal chloroform standard with a chemical shift value of 77.2 ppm. Assignments of identity were made on the basis of ^{13}C chemical shifts (17) and by comparing experimental values to the 3-methyl butyric acid-1-^{13}C standard (Isotec, Inc.).

Genome analysis. The genome of *B. linens* ATCC 9174 was sequenced by the Joint Genome Institute (JGI) to produce a draft sequence with at least 10× coverage. This sequence was autoannotated at Oak Ridge National Laboratory (ORNL), and the draft genome was posted at http://www.jgi.doe.gov/JGI_microbial/html/index.html for public access. In this work the genome was browsed and queried using the tools available at the JGI and ORNL (http://www.ornl.gov/sci/microbialgenomes/organisms.shtml) websites to determine the occurrence of the specific genes related to FA metabolism, which were visualized by using a Kyoto Encyclopedia of Genes and Genomes (KEGG [http://www.genome.ad.jp/kegg]) map.

Statistical analysis. FA determinations were replicated twice, and the data were averaged before presentation. Student's *t* test was used to determine significant differences in FA production among the organisms and substrates. Results with α values of ≤0.05 were considered significantly different for FA production. Standard deviation for FA production was ≤10% for all products.

RESULTS

End product accumulation. FA production was determined using GC and NMR analyses with cells that were exposed under carbohydrate-complete (TSB) or -depleted (CDM) conditions in this study. An assay system that mimics the salt and pH of the water phase of cheddar cheese was used for all substrates, primarily because keto acids are unstable for extended incubations (8). However, this approach was also used to avoid confounding interactions between multiple substrates from a complex medium for the production of FAs. All data are reported as incubation times for the culture, not the assay conditions.

Preliminary analysis of culture supernatants from logarithmic-phase growth under carbohydrate-replete conditions found FA production from α-keto acids (Fig. 1) but failed to detect FA products from amino acids (data not shown). All substrates resulted in the production of acetic acid predominantly. Propionic acid was detected with KIC and KIC with pyruvate added only. KIC also led to the production of isovaleric acid. Subsequently, BL2 was incubated under carbohydrate starvation conditions for ~7 days prior, which proved successful for the production of FAs from all substrates tested. Concurrently, catabolism of [^{13}C]leucine was determined using NMR spectroscopy.

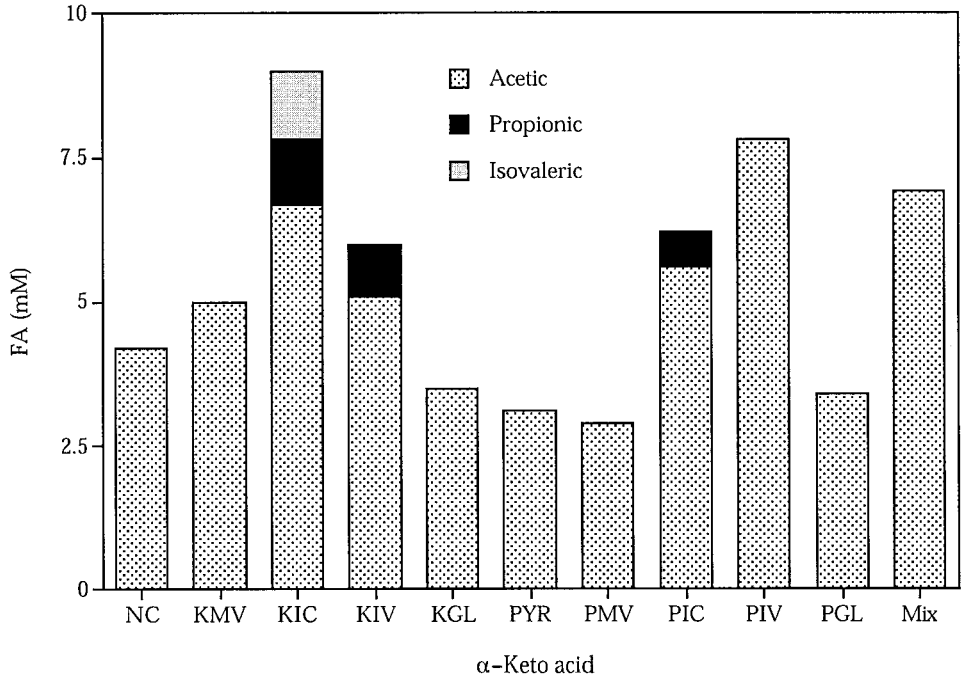

FIG. 1. Mean FA production from α-keto acids by cells from carbohydrate-complete medium (TSB). NC, negative control (cells in plain buffer); Pyr, pyruvate; PMV, pyruvate plus KMV; PIC, pyruvate plus KIC; PIV, pyruvate plus KIV; PGL, pyruvate plus KGL; Mix, mix of the above five α-keto acids. The standard deviation averaged 4.9% for all analytes tested.

In consideration of these observations, carbohydrate-depleted conditions were used to grow cells and the FA production was assayed. α-Keto acids also served as substrates for FA production, except the type of products increased (Fig. 2A). Acetic acid production declined in comparison to that under carbohydrate-complete conditions, while propionic and isovaleric acid production increased. Isobutyric and caproic acids were also found. The addition of pyruvate along with the α-keto acids reduced the quantities of BCFAs produced from KIC and KIV, but not from KMV.

To verify the production of FAs from amino acids, a time series experiment was done directly from the carbohydrate-depleted CDM. Leucine and valine were added to CDM prior to inoculation with BL2. The inoculated medium was incubated for 5 to 7 days at 25°C with aeration. During the incubation time, BL2 produced FAs from each amino acid (Fig. 2A). With Leu as the substrate, isovaleric acid was the predominant end product, with acetic acid being produced at an ~1 mM concentration over the duration of the incubation time (Fig. 2B). *n*-Butyric acid was not found at measurable concentrations during the testing period. Interestingly, isovaleric acid reached a peak concentration of ~1.75 mM at 48 h and then declined to undetectable concentrations at 160 h.

With the addition of Val to CDM as the substrate, BL2 produced the maximum FAs within 24 h (Fig. 2C). A new product, 4-methyl-*n*-valeric acid, was found under this condition, while isovaleric acid was not. All products declined over time, except acetic acid. Compared to Leu as the substrate, *n*-butyric acid production tripled and propionic acid concentration doubled.

The metabolic end products were verified using NMR analysis. Subsequently, the FA products were metabolized to below the detection limit during 120 to 168 h (5 to 7 days) of incubation. Identification of intermediate compounds in the metabolic route of Leu to FAs was not achieved. However, end product accumulation was determined to verify the production in time series experiments and α-keto acid catabolism assays.

Pathway estimation via genome analysis. The draft genome of *B. linens* ATCC 9174 (http://www.jgi.doe.gov/JGI_microbial/html/index.html) was examined for the presence of the genes required for the conversion of amino acids to FAs as described by various pathways in KEGG. Unlike lactococci (8, 9), *B. linens* contained all the genes needed for the transformation of amino acids to FAs via FA synthesis pathway 1 (KEGG map00061), FA biosynthesis pathway 2 (KEGG map00062), and the FA metabolism pathway (KEGG map00071) (see Fig. 4, below). Other interacting pathways included butanoate metabolism (KEGG map006650) and 1-carbon pool by folate (KEGG map00670), which are contained in part by *B. linens* but neither is included in the *Lactococcus lactis* subsp. *lactis* IL-1403 (http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db = genome&gi = 171) or the *L. lactis* subsp. *cremoris* SK11 (http://www.jgi.doe.gov/JGI_microbial/html/index.html) genomes. The FA metabolism pathways in *B. linens* have specific genes for each catabolic step that is differentiated by the FA chain length. Considering all the FA metabolic maps, this represents over 100 genes, some of which have multiple homologues. The genome of *B. linens* contains the genes needed for each step in FA biosynthesis and each step in the catabolism for energy. For example, in the FA metabolic pathway, butanoyl coenzyme

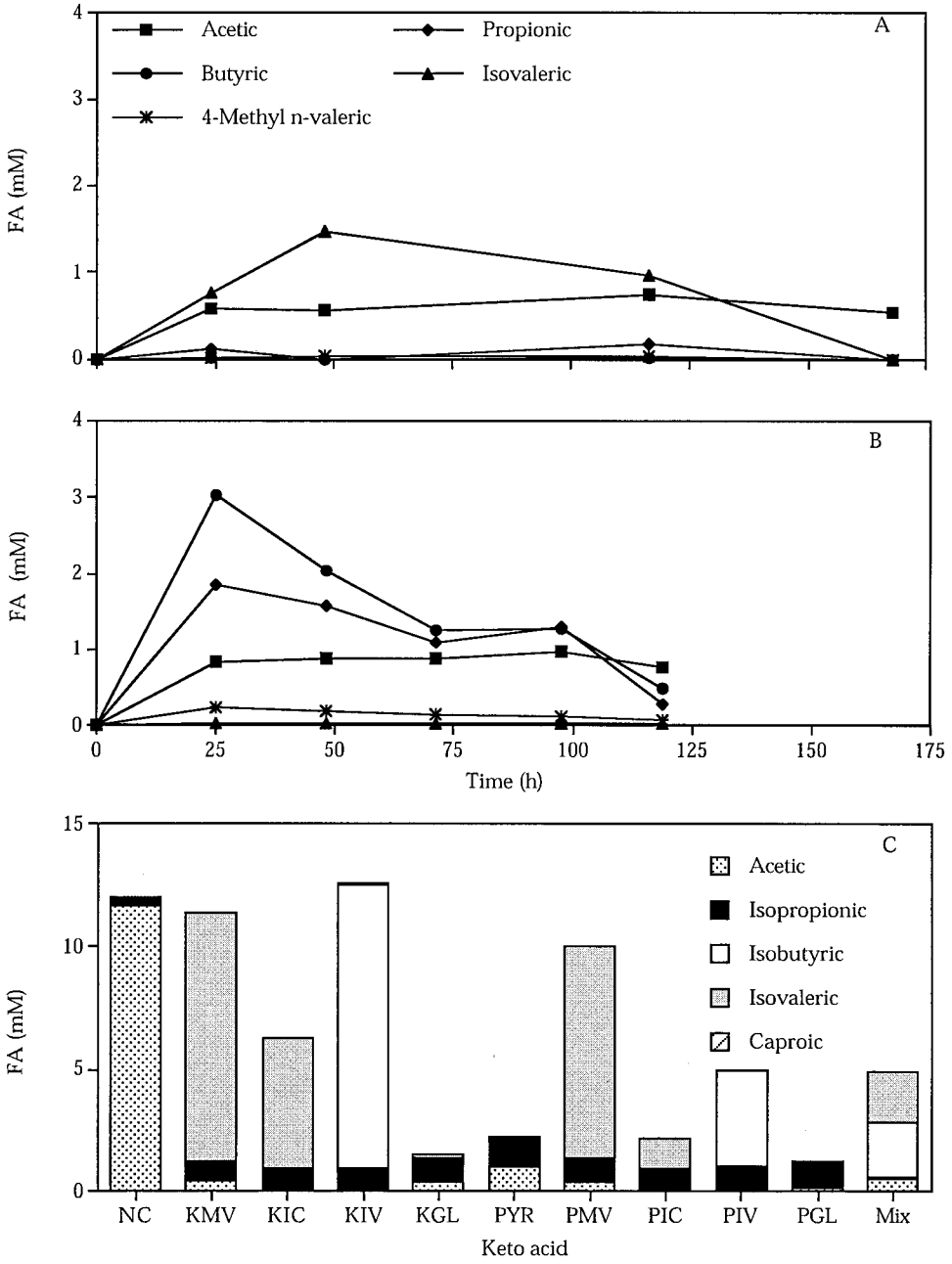

FIG. 2. FA production from carbohydrate-starved cells of *B. linens* BL2. Production of FAs from leucine (A) in a time series during incubation in CDM, production of FAs from valine in a time series during incubation in CDM (B), and FA production from keto acids during the 3-h assay (C). NC, negative control (cells in plain buffer); Pyr, pyruvate; PMV, pyruvate plus KMV; PIC, pyruvate plus KIC; PIV, pyruvate plus KIV; PGL, pyruvate plus KGL; Mix, mix of the above five α-keto acids. FA concentration was determined by GC analysis under all conditions. The standard deviation averaged 2.6% for all analytes tested.

A (CoA) is converted to *trans*-butanoyl-2-enoyl-CoA via three different acyl-CoA dehydrogenase enzymes (EC 1.3.3.6, 1.3.99.2, and 1.3.99.3). All of these genes are present in the *B. linens* genome. These defined pathways suggest that *B. linens* has all the genes needed for the production of FAs from amino acids and for the reassimilation of the FAs as an energy source and oxidation-reduction regulation mechanism, as demonstrated during the time course experiment (Fig. 2A and B).

DISCUSSION

Brevibacteria accelerate flavor development in low-fat cheddar cheese when added with the starter culture via the production of sulfur and FAs (1, 10, 20). This change is associated with the later stages of cheese ripening, which correspond to lactose depletion in cheese. However, detailed information related to the mechanism of FA production is very limited in

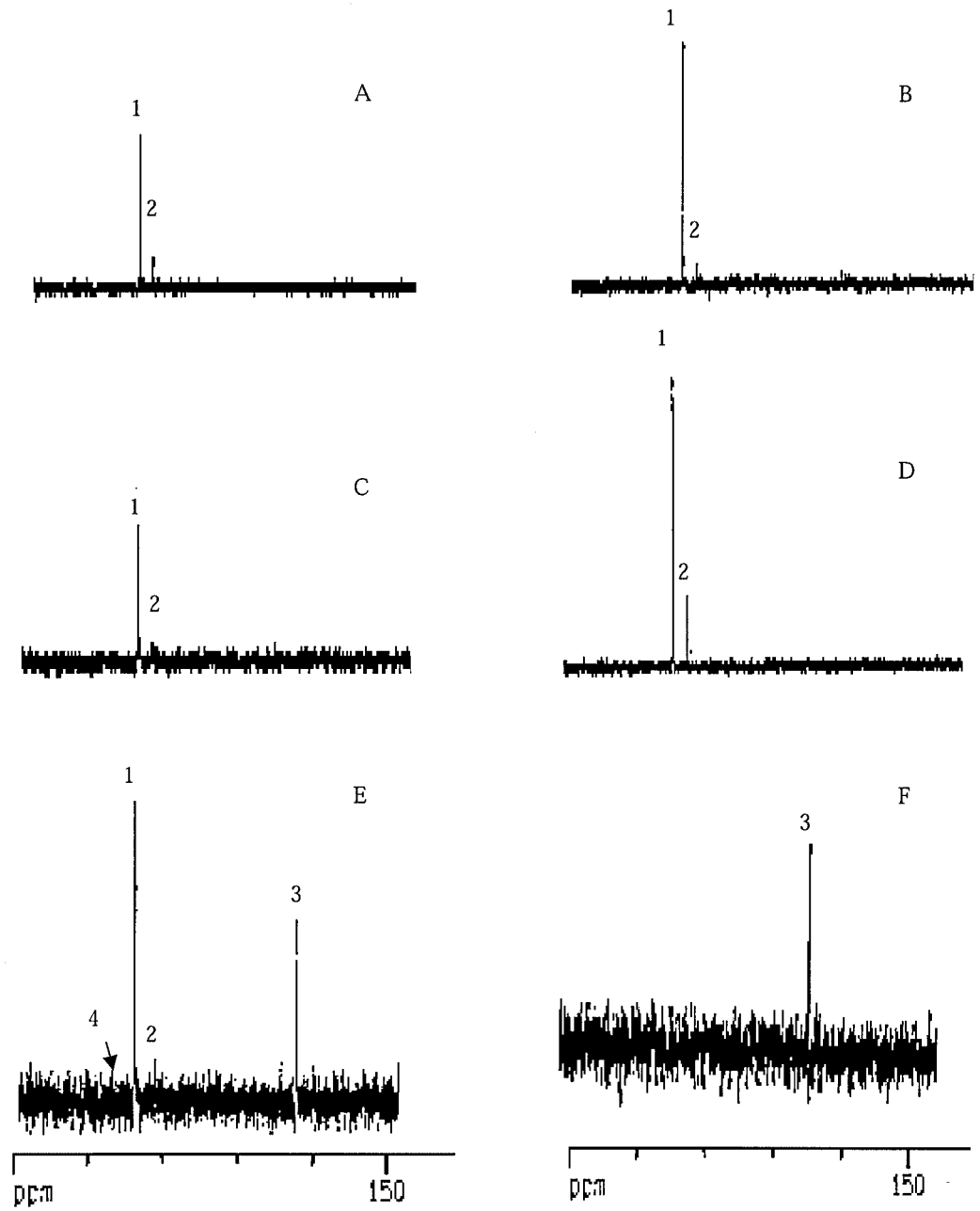

FIG. 3. Determination of FA production by NMR spectroscopy using Leu as the substrate. The scans (in order from left to right, top to bottom) represent products detected at 48 (A), 71 (B), 96 (C), 120 (D), 144 (E), and 168 (F) h, respectively. Peaks on the spectra: 2, C-1 of isovaleric acid, 179.98 ppm; 2, C-1 of acetic acid, 178.13 ppm; 3, C-3 of KGL, 162.07 ppm; 4, C-1 of propionic acid, 181.12 ppm.

bacteria. Coupled to the limited information and the connection to greater influence during the later stages of ripening, we studied the ability of *B. linens* BL2 to produce BCFAs from BCAAs and α-keto acids after the pure culture entered stationary phase and the carbohydrate was depleted. Our initial observations confirmed the initial observations of Hosono (13) for FA production. Additionally, we demonstrated that FAs were consumed by BL2 during longer incubation times. Our observations, along with previous studies (13), indicate that brevibacteria are capable of producing FAs from amino acids, but only after carbohydrate depletion. This is reasonable based on the need for metabolic energy to activate BCAA catabolism (11) to produce ATP via substrate-level phosphorylation. Delineation of the metabolic pathway (9) and gene expression regulation is required to determine the mechanism for production of FAs from amino and keto acids.

Catabolism of the BCAAs leucine and valine during the assay incubation produced FAs at different concentrations, though they were of similar types (Fig. 2). Leucine was catabolized to isovaleric acid, which is the end product that would be expected from the predicted pathway of BL2 (9), while valine catabolism resulted in *n*-butyric acid rather than the expected

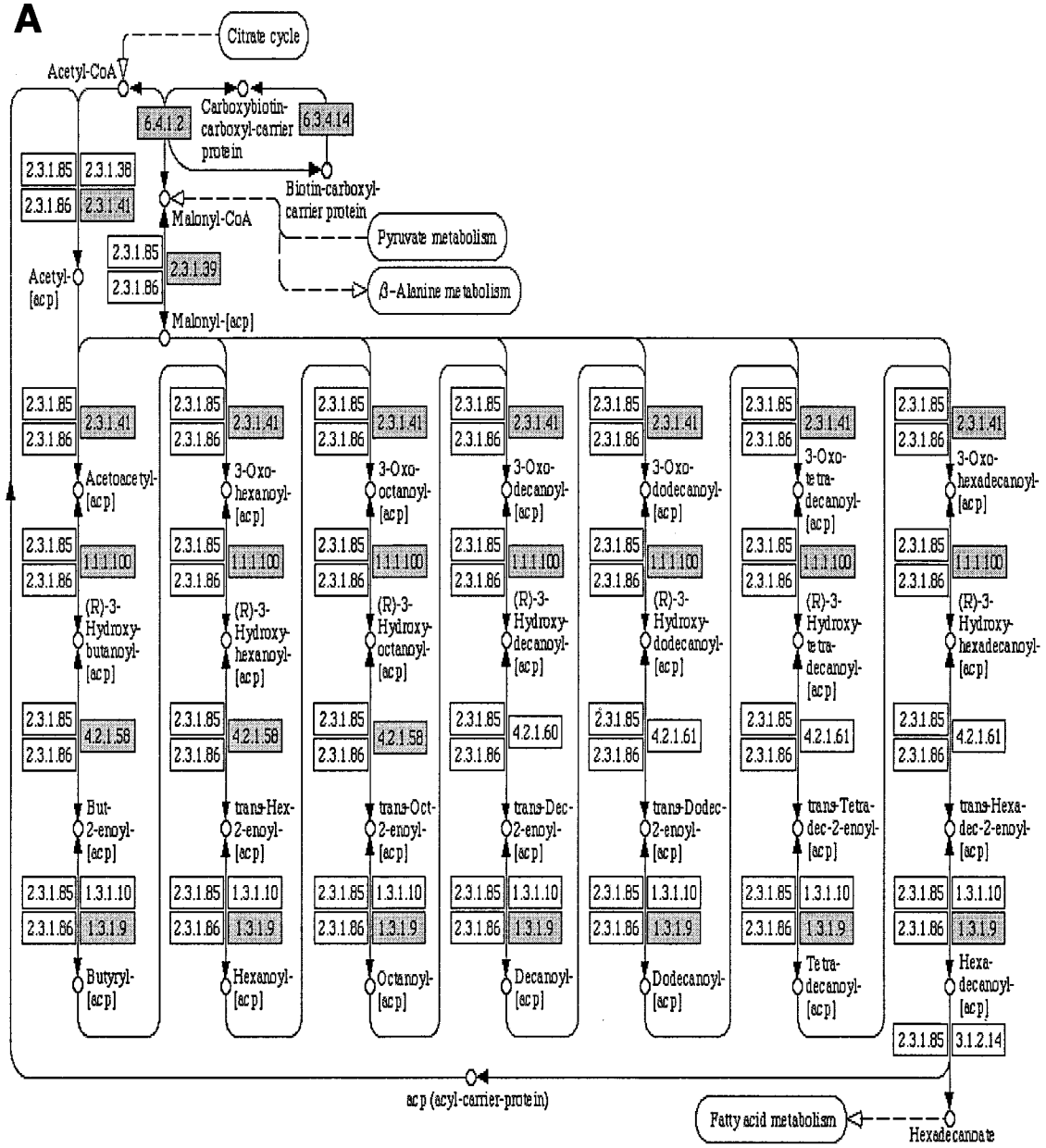

FIG. 4. Genes associated with the metabolic fate of FAs via biosynthesis (A), via biosynthesis through an alternate pathway (B), or during metabolism (C) in the *B. linens* ATCC 9174 genome. The shaded boxes represent the gene products that are present in the genome.

product, isobutyric acid. This observation was unexpected and is difficult to explain via known metabolic pathways. One explanation may be due to the lax ATase substrate specificities in lactococci (8, 9). Alternatively, intermediates that lead to isobutyric acid may have been catabolized via alternate pathways to acetyl-CoA and were subsequently condensed to produce *n*-butyryl-CoA and, hence, *n*-butyric acid. The genes encoding the enzymes for these pathways exist in the genome of BL2 (http://www.jgi.doe.gov/JGI_microbial/html/index.html). Transcription of these genes is unknown and must be characterized to prove this possible explanation.

Previous studies in lactococci (9, 23, 24) demonstrated that catabolism of BCAAs or their corresponding α-keto acids differs mechanistically due to the interaction of complex webs of multiple ATases. Studies in brevibacterial catabolism of amino acids are currently limited to aromatic and sulfur-containing amino acids (19), making it difficult to assess the substrate specificities of ATases in this organism. In order to determine the role of ATases in BCFA production in brevibacteria, this study tested catabolism of α-keto acids by BL2, hence bypassing the ATase reaction to alleviate the confounding factors in substrate specificity of these enzymes.

Metabolism of α-keto acids did not require carbohydrate starvation and resulted in acetic and propionic acids in logarithmic-phase cultures. The α-keto acids were transported and metabolized to FAs within the 3-h incubation, suggesting that

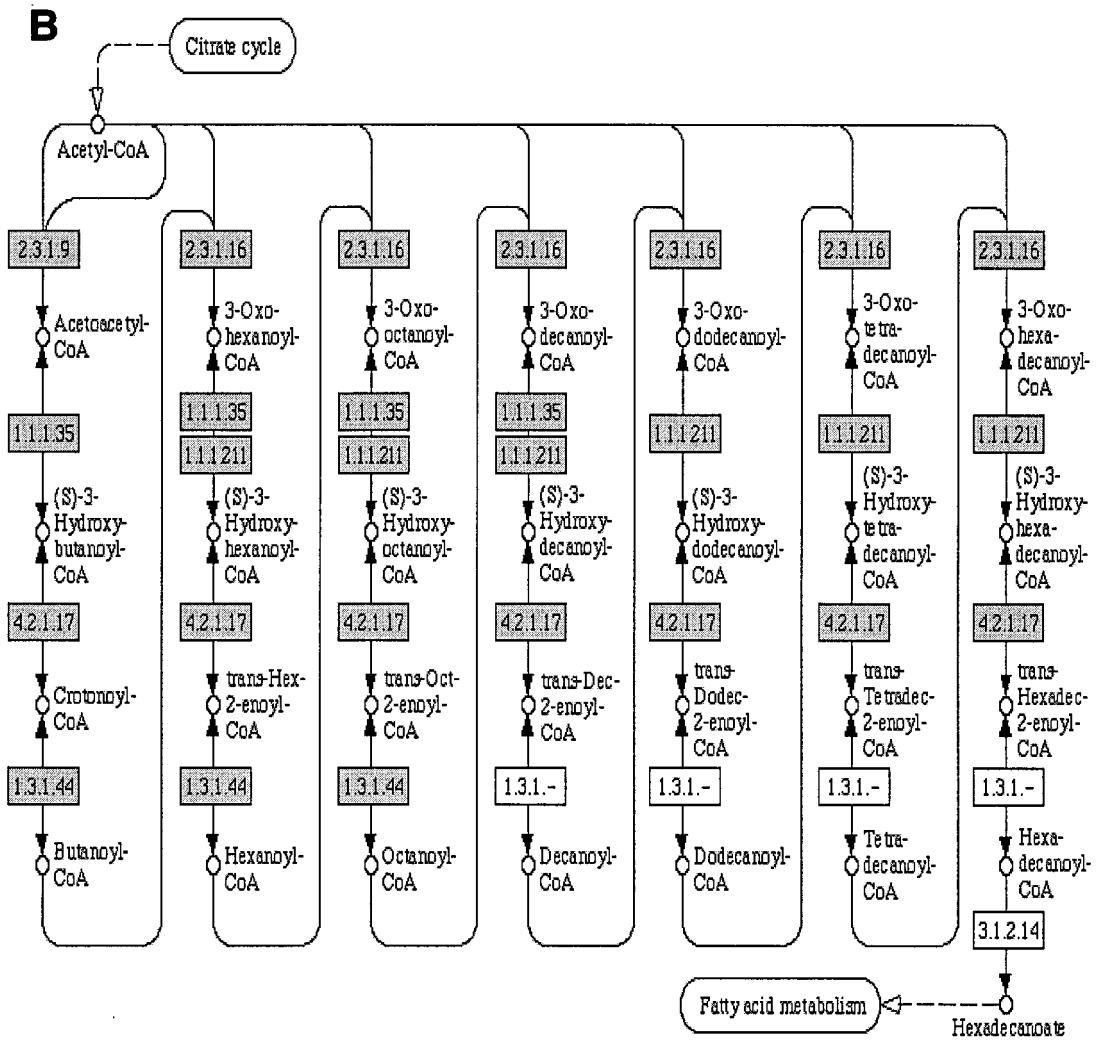

FIG. 4—*Continued*.

either amino acid transport or ATase expression is controlled by the carbohydrate status of the cell. Alternatively, log-phase cells may utilize the α-keto acids via other metabolic pathways, while acetic and propionic acids are produced from other energy-yielding intracellular intermediates, such as pyruvate or acyl-CoAs. The verification of these explanations was beyond the scope of this study.

Carbohydrate-starved cells of BL2 also catabolized α-keto acids to yield BCFAs exclusively, as with amino acids (Fig. 3). This is contrary to the metabolism of lactococci and lactobacilli (9). Interestingly, BL2 subsequently catabolized FAs after their production from amino or keto acids. Presumably, the FAs were catabolized via β-oxidation and channeled toward the tricarboxylic acid cycle and other energy-producing pathways, redox-reactive compounds, and other shorter-chain FAs (Fig. 3).

Addition of pyruvate to KIC and KIV reduced the amount of BCFAs produced via α-keto acid catabolism in assays with carbohydrate-starved cells, providing further evidence for the importance of carbohydrate status in this metabolism. An FA product profile change was also observed in assays with logarithmic-phase cells. This may be due to the energetic state of the cell modulated by presence of pyruvate, which can be catabolized to products other than FAs (9, 18). The intracellular abundance of these metabolic intermediates may explain why log-phase cells of BL2 did not catabolize amino acids to FAs.

Investigation of the genome of *B. linens* ATCC 9174 found that this organism has all the genes required to produce and degrade FAs (Fig. 4). These consist of three primary pathways, two of which are anabolic and the other is catabolic. Each pathway was compared via KEGG between *B. linens* ATCC 9174, *Escherichia coli* K-12 W3110, *Bacillus subtilis*, *Bacillus cereus*, *Corynebacterium glutamicum*, *Corynebacterium efficiens*, *Corynebacterium diphtheriae*, *L. lactis* subsp. *lactis* IL-1403, *Listeria monocytogenes* F2365, and *Lactobacillus plantarum* WCFS1. FA pathway 1 was the most conserved, with all the genes being present, except that those in *E. coli* K-12, *B. subtilis*, and *B. cereus* were missing the dehydroxylase (EC 4.2.1.58). All the organisms other than *B. linens* were missing FA pathway 2. In the case of the lactic acid bacteria, they were missing all the enzymes. This was also the case with FA me-

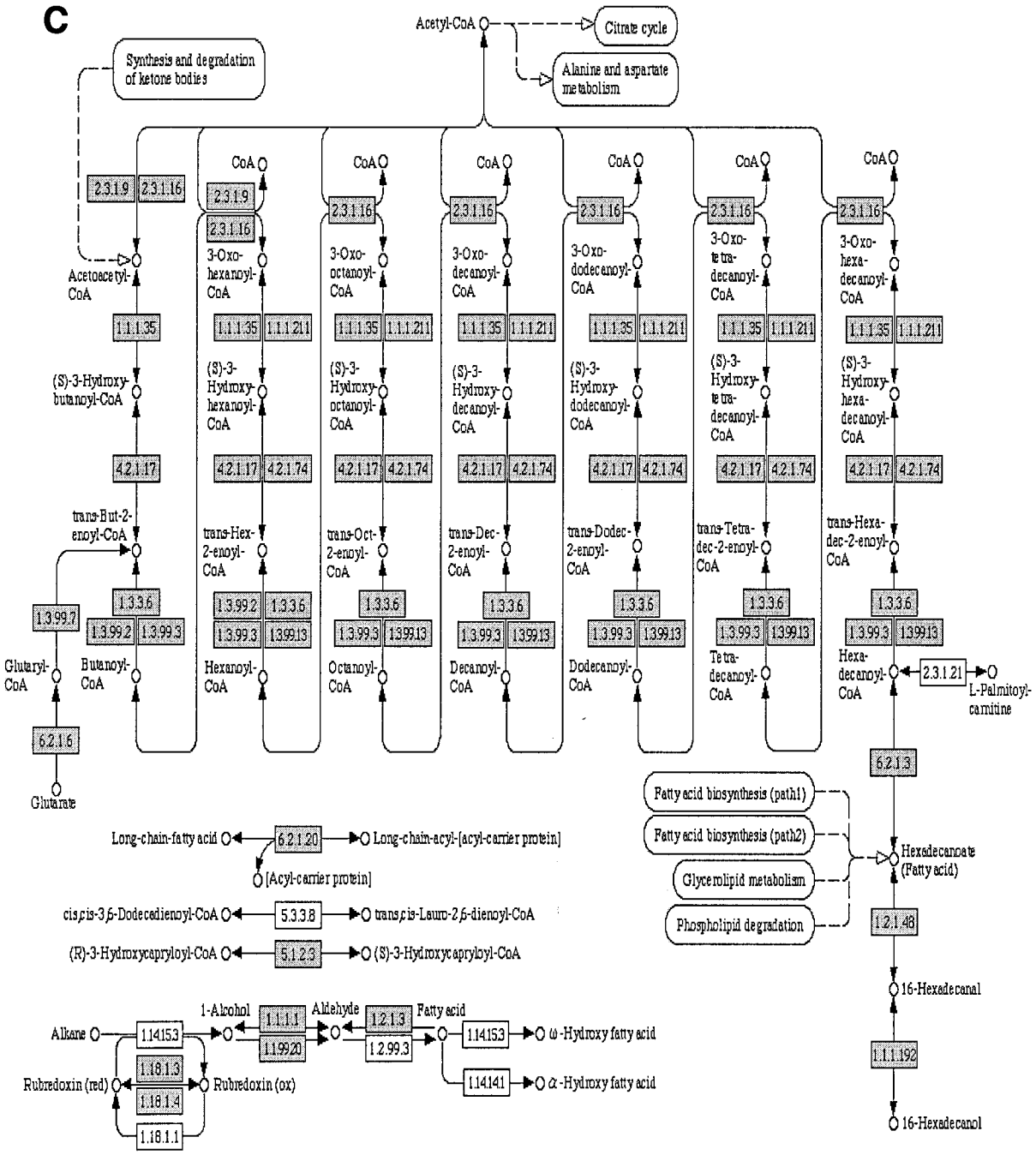

FIG. 4—*Continued*.

tabolism. None of the organisms other than *B. linens* contained the genes for the complete pathway. This observation makes *B. linens* somewhat unique among a diverse set of organisms and closely related coryneform bacteria. Further work is needed to define the exact genes and proteins that are used to produce and degrade FAs in this organism.

The observations of this study provide evidence for the production of BCFAs from amino acids and α-keto acids by brevibacteria and their further catabolism to other FAs. This study has also established the importance of carbohydrate status to control amino acid catabolism and the independence of α-keto acid catabolism to FAs from this control. These observations indicate that brevibacteria have an intricate mechanism that regulates the production of FAs from protein sources. Presumably, the driving force is related to the energy status of the cell.

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Mention of companies and products does not constitute endorsement by Utah State University or Utah Agricultural Experiment Station over similar products not mentioned.

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